Analysis of Rhubarb by Liquid Chromatography-Electrospray-Mass Spectrometry

Ming-Ren S. Fuh* and Hung-Jian Lin

Department of Chemistry
Soochow University
Taipei, Taiwan 111, R.O.C.
E-mail: msfuh@mail.scu.edu.tw

Abstract

Liquid chromatography-electrospray-mass spectrometry (LC-ES-MS) was employed to analyze five biologically active components (sennoside A and B, rhein, emodin and aloe-emodin) in rhubarb. The separation was achieved on a C18 column using gradient elution. Negative ion detection mode was utilized for ES-MS detection. For sennosides, [M-H]− and a fragmented ion (m/z = 386) were detected. In addition to [M-H]−, [M-COOH]− was observed for rhein. Only one major ion, [M-H]−, was determined for aloe-emodin and emodin. A post-column splitter was employed to enhance the sensitivity of sennoside measurement. Extracted ion monitoring was applied for quantitative analysis. Satisfactory linearity (r² = 0.99) of each compound was determined. In addition, the content of these five active components in various rhubarb samples were examined by this newly developed LC-ES-MS.

Key Words: LC-MS, Chinese Herbal Medicine, Sennoside

1. Introduction

Rhubarb is commonly used for purgative crude drug in traditional Chinese medicine. Sennosides and anthraquinones (aleo-emodin, emodin, rhein and so on) were identified as the biologically active compounds in rhubarb [1,2,3]. Several methods have been reported for the determination of the constituents of rhubarb, including thin-layer chromatography [4,5], high performance liquid chromatography [6-10] and capillary electrophoresis [11].

Liquid chromatography-electrospray-mass spectrometry (LC-ES-MS) has emerged as a sensitive and accurate analytical technique. Electrospray generates ions under atmospheric pressure and at relatively low temperature which minimizes thermal decomposition of labile compounds. In addition, mass spectrometry offers highly selective measurement by detecting specific mass-to-charge (m/z) ion related to analytical component; hence, more precise assignment of each eluted component. LC-ES-MS provides the capability to determine target compounds in complex matrix. It has proved to be very effective for the analysis of various compounds [12-16].

This study evaluates the effectiveness of LC-ES-MS for the analysis of the major components (sennoside A, sennoside B, rhein, aloe-emodin and emodin) in rhubarb. Collision-induced dissociation was examined and optimized for each compound. The enhancement of post-column flow splitting on the ES-MS detection was investigated. In addition, the constituents of various rhubarb samples were examined by this newly developed method.
2. Experimental

2.1 Chemicals

HPLC grade acetonitrile and methanol (Mal- linckrodt Baker, Inc., Paris, KY, U.S.A.) and HPLC grade water (Labscan Limited, Dublin, Ireland) were used throughout the experiment. Acetic acid and ammonia solution were obtained from Nacalai Tesque (Kyoto, Japan). Sennoside A and B were purchased from Extrasynthese (Genay, France). Rhein, aloe-emodin and emodin were from Sigma (St. Louis, MO, U.S.A.). Various rhubarb samples were donated by Brion Research Institute of Taiwan (Taipei, Taiwan).

2.2 HPLC System

An HP1050 quaternary pump was used for LC (Hewlett-Packard Co., Palo Alto, CA, U.S.A.). A LiChroCART RP-18e column (Purospher, 125 x 3 mm, 5 µm, Merck, Darmstadt, Germany) with an LiChroCART 4-4 on-line guard column was used for separation. The mobile phase was 0.015% acetic acid (solution A) and acetonitrile containing 0.015% acetic acid (solution B). The flow rate was 0.5 mL/min. and the injection volume was 20 µL. The gradient elution condition is summarized in Table 1. After completing the chromatographic elution, the mobile phase was programmed to its initial condition within 10 min. and a 15 min. re-condition time was set before next injection.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>80</td>
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<td>16</td>
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<td>55</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>75</td>
<td>85</td>
<td>15</td>
</tr>
</tbody>
</table>

2.3 Mass Spectrometry

An HP-5988B mass spectrometer with a HP-59987A electrospray interface (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used. An HP Chemstation (G1034C, version C.03.00) was utilized for system control, data acquisition and data analysis. Heated N₂ gas (350 °C, 12.5 mL/min.) and O₂ gas (350 °C, 6.3 mL/min.) was used to evaporate solvent from the electrospray chamber and compressed N₂ gas (80 psi) was used for nebulization. The cylinder electrode in the electrospray chamber was set at 3500 V. The end plate and capillary entrance voltage were set at 3000 V and 3500 V. The voltage of skimmer 1, lens 1, skimmer 2, lens 2 and lens 3 were set at −45.5 V, 0.6 V, −8.2 V, −14.0 V and 84 V, respectively. The mass spectrometer was operated at negative ion mode and mass spectra collected in scan mode were obtained by scanning from 50 to 1000 in 0.5 seconds. Nine scans were averaged with a step size 0.1 over the range. The mass spectrometer was tuned with the procedures provided by Hewlett-Packard Company (17). The tuning mixture consisting of valine (m/z = 116), tri-tyrosine (m/z = 506) and hexa-tyrosine (m/z = 995) was obtained from the same company.

2.4 Sample Preparation

Finely ground powder rhubarb (about 0.5 g) was extracted with 70% methanol (each 5 mL) by stirring at room temperature for 30 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter. The solution was stored in the refrigerator (4 °C). A micro-porous filter (0.45 µm) was utilized to filter the solution prior to LC-MS analysis.

3. Results and Discussion

Electrospray is a soft-ionization technique, collision-induced dissociation (CID) has been used to enhance molecular fragmentation. Negative ion mode was employed for the detection of these compounds. For sennoside A and B, [M-H]⁻ and a major fragmented ion (m/z = 386) were detected. Two major ions of rhein, [M-H]⁻ and [M-COOH]⁻, were measured. For aloe-emodin and emodin, only one major ion, [M-H]⁻, was observed. The structures of these five compounds and proposed fragmented ions are shown in Figure 1. Furthermore, the abundances of these ions were generally dependent upon CID voltage. For sennoside A and B, the abundance of [M-H]⁻ ion gradually increased (about 25%) when the CID voltage changed from −75 V to −200 V. However, the abundance of the major fragmented ion (m/z = 386) increased significantly (about 230%) when the CID voltage reduced from −75 V to −125 V, but diminished substantially while CID voltage went beyond −125 V. For rhein, the intensities of both [M-H]⁻ and [M-COOH]⁻ ions increased while the CID voltage changed from −75 V to −125 V and decreased as the voltage passed −125 V. For aloe-emodin and emodin, the abundance of [M-H]⁻
ion increased while CID voltage diminished from $-75 \text{ V}$ to $-150 \text{ V}$ and decreased as the voltage went beyond $-150 \text{ V}$. In this study, CID voltage was set at $-125 \text{ V}$ for the detection of sennosides and rhein, and at $-150 \text{ V}$ for aloe-emodin and emodin. The negative ES ionization mass spectra of these compounds are shown in Figure 2.

Figure 1. Structures of the studied compounds

Figure 2. Negative ES ionization spectra of studied compounds

Figure 3. LC-ES-MS chromatogram of standard solution, (a) TIC: total ion chromatogram; (b) to (f) extracted ion chromatogram; 1. sennoside B, 2. sennoside A, 3. rhein, 4. aloe-emodin, 5. emodin

A liquid chromatography method utilizing a C$_{18}$ column and gradient elution was developed to separate these five components. Small amount of acetic acid was needed in mobile phase to separate these compounds. However, the addition of acidic modifier in mobile phase reduces the ES-MS abundance (~10%). LC-ES-MS total ion chromatogram and the extracted ion chromatograms of the five standard compounds are shown in Figure 3. For sennosides, two extracted ion ([M-H]$^-$ and [m/z = 386]) were used for identification and the primary ion (m/z = 386) was used for quantitative measurement. [M-H]$^-$ and [M-COOH]$^-$ ions were employed to detect rhein and the [M-COOH]$^-$ ion was used for quantitative determination. However, only [M-H]$^-$ ion was utilized in the detection of aloe-emodin and emodin.
The flow rate of sample introduction for ES-MS is also an important parameter affecting the sensitivity of LC-ES-MS detection [15,17,18]. A splitting-T between LC separation column and the entrance of ES-MS was placed to adjust sample introduction rate and examine the effect of flow-rate on the LC-ES-MS detection. The results of this study were summarized in Figure 4. For sennosides, the intensities increase considerably when the introduction rate reduced from 0.5 to 0.11 mL/min. It might be attributed to poor nebulization efficiencies for sennosides since it eluted at higher water content; thus, fewer ions were generated when higher flow rate was employed. Less significant effect of flow rate on LC-ES-MS response was determined for rhein and aloe-emodin. However, reversed trend was observed for emodin. Emodin eluted at higher acetonitrile concentration and good nebulization efficiency was achieved; therefore, less amount of sample molecule was introduced into ES-MS when the introduction rate was reduced. Post-column split flow was applied in this study. At this flow rate (0.11 mL/min.), the sensitivity of sennosides increased approximately 3 folds while the sensitivity of emodin only reduced about 25%.

The linearity of this newly developed method was evaluated by analyzing a series of standards. Quantitative results were obtained by extracted ion monitoring. The results of this study were summarized in Table 2. Adequate linearity ($r^2 = 0.99$) was obtained through the range examined. The detection limit, based on a signal-to-noise ratio of 3, was ranged from 0.04 to 0.4 ppm. Emodin, aloe-emodin and rhein appeared to have much better sensitivity than sennosides.

In order to examine the matrix effect of medicinal plant extract on the determination of these compounds, a suitable amount of the five compounds were added into a sample extract and then analyzed by LC-ES-MS. The results are summarized in Table 4. The recoveries of these five compounds were ranged from 94.6 to 98.2 %. It demonstrated that this sample extraction procedure is effective and indicated that there is no matrix effect on the measurement. The application of this newly developed LC-ES-MS method was demonstrated by evaluating these five components in various rhubarb samples. A typical LC-ES-MS chromatogram of rhubarb extract is shown in Figure 5. There were many components detected in total ion chromatogram (Figure 5(a)). Nevertheless, the five target compounds could be identified and quantified by extracted ion chromatograms (Figure 5(b) to 5(f)). The results of several rhubarb samples examined by this newly developed method are summarized in Table 4.

In summary, this work has successfully demonstrated the potential of LC-ES-MS for quantitative determination of sennoside A & B, rhein, aloe-emodin and emodin in rhubarb. Adequate linearity and detection limit were also obtained. In addition, the application of this newly developed method was demonstrated by analyzing various rhubarb samples. LC-ES-MS method is a promising alternative to the analysis of other traditional Chinese herbal medicine.

![Figure 4. Effect flow rate on LC-ES-MS response](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ret.(^a) time, min.</th>
<th>Calibration equation (^b)</th>
<th>Linear range, $\mu$g/mL</th>
<th>$r^2$</th>
<th>D.L.(^c), ppm</th>
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</thead>
<tbody>
<tr>
<td>sennoside B</td>
<td>5.4</td>
<td>$Y = 59616X + 205617$</td>
<td>2-100</td>
<td>0.99</td>
<td>0.4</td>
</tr>
<tr>
<td>sennoside A</td>
<td>9.0</td>
<td>$Y = 100413X + 363418$</td>
<td>2-100</td>
<td>0.99</td>
<td>0.4</td>
</tr>
<tr>
<td>rhein</td>
<td>24.1</td>
<td>$Y = 8753516X + 1485639$</td>
<td>0.2-20</td>
<td>0.99</td>
<td>0.06</td>
</tr>
<tr>
<td>aloe-emodin</td>
<td>26.3</td>
<td>$Y = 1898894X + 737866$</td>
<td>0.5-50</td>
<td>0.99</td>
<td>0.06</td>
</tr>
<tr>
<td>emodin</td>
<td>34.5</td>
<td>$Y = 24940615X + 4449332$</td>
<td>0.1-10</td>
<td>0.99</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\(a\). retention time.

\(b\). X-conc. ($\mu$g/mL); Y-peak area

\(c\). D.L.-detection limit (3 times of signal-to-noise ratio)
Table 3. Summary of recovery study

<table>
<thead>
<tr>
<th>amount spiked^a</th>
<th>sennoside A</th>
<th>sennoside B^a</th>
<th>rhein</th>
<th>aloe-emodin</th>
<th>emodin^a</th>
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<tbody>
<tr>
<td>recovery^b</td>
<td>97.6%</td>
<td>91.1%</td>
<td>99.6%</td>
<td>96.4%</td>
<td>99.0%</td>
</tr>
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</table>

a. µg/mL
b. average of three measurements

Table 4. Results of various rhubarb samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>sennoside A^a</th>
<th>sennoside B^a</th>
<th>rhein^a</th>
<th>aloe-emodin^a</th>
<th>emodin^a</th>
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<tbody>
<tr>
<td>Sample-1</td>
<td>660</td>
<td>264</td>
<td>2165</td>
<td>2501</td>
<td>1661</td>
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<tr>
<td>Sample-2</td>
<td>5058</td>
<td>2281</td>
<td>2461</td>
<td>3119</td>
<td>1756</td>
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<tr>
<td>Sample-3</td>
<td>6073</td>
<td>10136</td>
<td>2114</td>
<td>5686</td>
<td>2973</td>
</tr>
<tr>
<td>Sample-4</td>
<td>6958</td>
<td>2265</td>
<td>2092</td>
<td>5628</td>
<td>2943</td>
</tr>
</tbody>
</table>

a. mg/kg
b. dilute 3 fold prior to LC-ES-MS analysis
c. dilute 5 fold prior to LC-ES-MS analysis

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References


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